Light regulates motility, attachment and virulence in the plant pathogen *Pseudomonas syringae* pv tomato DC3000

Isabel Río-Álvarez, José Juan Rodríguez-Herva, Pedro Manuel Martínez, Pablo González-Melendi, Gloria García-Casado, Pablo Rodríguez-Palenzuela and Emilia López-Solanilla

Summary

***Pseudomonas syringae* pv tomato DC3000 (Pto) is the causal agent of the bacterial speck of tomato, which leads to significant economic losses in this crop. Pto inhabits the tomato phyllosphere, where the pathogen is highly exposed to light, among other environmental factors. Light represents a stressful condition and acts as a source of information associated with different plant defence levels. Here, we analysed the presence of both blue and red light photoreceptors in a group of *Pseudomonas*. In addition, we studied the effect of white, blue and red light on Pto features related to epiphytic fitness. While white and blue light inhibit motility, bacterial attachment to plant leaves is promoted. Moreover, these phenotypes are altered in a blue-light receptor mutant. These light-controlled changes during the epiphytic stage cause a reduction in virulence, highlighting the relevance of motility during the entry process to the plant apoplast. This study demonstrated the key role of light perception in the Pto phenotype switching and its effect on virulence.**

Introduction

Light and circadian cycle regulate plant defence mechanisms (Roden and Ingle, 2009; Bhardwaj *et al.*, 2011; Wang *et al.*, 2011; Kangasjarvi *et al.*, 2012). Plant pathogens might have evolved to sense light conditions associated with different levels of plant resistance. Indeed, bacteria possess mechanisms for the detection of light, which changes the motile behaviour (phototaxis) and other physiological processes for adaptation to the environment (Armitage and Hellingwerf, 2003; Purcell and Crosson, 2008). The effect of light has been extensively studied in phototrophic bacteria and only recently examined in non-phototrophic bacteria (Elias-Arnanz *et al.*, 2011), suggesting that the use of light as a source of information might be an evolutionary advantage. One of the reasonable explanations to consider is that this ability has an advantage in the development of a protective program against certain harmful light wavelengths, but recent reviews have shown that in many bacteria, light governs other important lifestyle decisions (Gomelsky and Hoff, 2011).

The bacterial photosensory protein modules for visible light include: (i) the blue light sensing light, oxygen and voltage LOV (light, oxygen and voltage), which belongs to the PAS (Per-Arnt-Sim) domain family, BLUF (blue-light sensing using FAD), PYP (photoactive yellow protein), and the cryptochrome/photolyase family; (ii) proteorhodopsins; and (iii) the red light sensing bacteriophytochromes. The light-sensing capacity of these proteins is mediated through associations with specific light-absorbing molecules or chromophores. Most of these photosensory proteins have a modular architecture, where the light-sensing input domain (e.g. LOV) can be combined with diverse output domains, such as histidine kinases (HKs), HATPase, EAL from phosphodiesterases, sulfate transporters anti-sigma factor antagonist, or response regulator (RR) and helix-turn-helix. The light-induced control of gene expression involves a wide gamut

of regulatory mechanisms associated with other stress responses (Elias-Arnanz *et al.*, 2011).

Although the photochemical properties of different types of photoreceptors have been analysed in some animal and plant pathogens (Lamparter *et al.*, 2002; Swartz *et al.*, 2007; Barkovits *et al.*, 2008; 2011; Rottwinkel *et al.*, 2010; Ondrusch and Kreft, 2011), the putative role of these proteins in the regulation of virulence remains poorly understood. Recent works show the involvement of photosensory proteins in the control of adaptive responses in *Agrobacterium tumefaciens* (Atu) (Oberpichler *et al.*, 2008), *Rhizobium leguminosarum* (Rle) (Bonomi *et al.*, 2012) and *Xanthomonas axonopodis* pv citri (Xax) (Kraiselburd *et al.*, 2012). These recent and interesting reports revealed a key role for blue light perception in the control of bacterial lifestyles.

Pseudomonas syringae pv tomato DC3000 (Pto) is a hemibiotrophic pathogen that multiplies in the apoplastic space exploiting live host cells. Pto causes bacterial speck in tomatoes and other plants, which is characterized by the development of necrotic symptoms in leaves, stems and fruits. Pto epiphytically and endophytically grows on plant foliage without causing disease symptoms (Hirano and Upper, 2000). The epiphytic lifestyle represents the initial phase of foliar colonization by Pto. During this stage, Pto is exposed to the atmosphere and subjected to the diurnal cycle. Eventually, Pto enters the plant leaf apoplast where it multiplies exploiting live host cells assisted by the type three secretion system that injects effector proteins into plant cells (Alfano and Collmer, 1997; Preston, 2000; Gohre and Robatzek, 2008; Block and Alfano, 2011).

During the life in the phyllosphere, bacteria feature specific adaptations to an environment characterized by nutrient limitation, fluctuating water availability, exposition to ultraviolet radiation, or the presence of antimicrobials from plants or other microorganisms (Vorholt, 2012). Bacteria actively move to favorable sites on leaf surfaces. *Pseudomonas syringae* has been shown to regulate motility by quorum signal mechanisms during the epiphytic stage (Quiñones *et al.*, 2005). Motility during this phase might be assisted by chemotaxis towards nutrients or plant molecules. Actually, Pto has been shown to be strongly attracted by plant origin molecules present in the tomato host plant (Cuppels, 1988). On the other hand, commensal bacteria found in the phyllosphere have developed specific adaptive mechanisms mainly devoted to adhere to the leaf surface and to produce EPS (Exopolysacharide) protective structures (Danhorn and Fuqua, 2007; Vorholt, 2012). It might be hypothesized that epiphytic fitness in a phytopathogenic bacteria would be determined by the ability to move looking for a favourable environment together with the ability to adhere to plant surface and to resist over the diurnal cycle the stressful situations imposed by the environment. The switch to a pathogenic lifestyle would require the entry to the plant apoplast, which is a motility dependent process. This switch might be dependent on light conditions. Interestingly, homologues to light receptors have been identified in Pto (van der Horst *et al.*, 2007). A Pto LOV protein exhibits light-regulated HK activity (Swartz *et al.*, 2007) and light-regulated communication between its various domains (Cao *et al.*, 2008). Moreover, the two Pto PHY proteins are active under red light *in vitro* (Shah *et al.*, 2012). However, no data concerning the putative role of these photosensory proteins in the pathogenesis have been reported.

In this study, we conducted a comparative analysis of the available *Pseudomonas* genomes to identify proteins containing LOV or PHY domains. In addition, we studied the effect of light on Pto phenotypes like motility and adhesion, and how this affects virulence. We observed that the blue light component is primarily responsible for the observed phenotypes. This conclusion was confirmed through an analysis of the phenotype resulting from a mutation in the LOV protein involved in blue light perception.

Taken together, these results show the specific control of white light over traits associated with Pto virulence.

Results

Plant-associated Pseudomonas *possess LOV and PHY photoreceptors*

Among the bacterial photosensory protein modules for visible light, blue light-sensing LOV proteins and red light-sensing bacteriophytochromes have been identified in many bacterial species. We have screened 33 *Pseudomonas* genomes for the presence of genes encoding these proteins, with respect to the phylogeny of the strains analysed, using a bioinformatics approach for the identification of the domains involved in this perception (Fig. 1, Tables S1 and S2). It has been previously suggested that the PAS family proteins, with the structure LOV-HK-RR, are predominately found in plant pathogenic bacteria (Losi and Gartner, 2008; 2011) and that these domains share important functional amino acid residues for photochemistry and signalling. Most of the *P. syringae* genomes analysed here possessed at least one protein with this structure, whereas other non-pathogenic *Pseudomonas*, even plant-associated bacteria, such as *P. fluorescens*, did not contain these proteins. The tomato pathogen Pto genome encodes 48 proteins containing PAS domains. Among these, only one protein presented the LOV-HK-RR structure. Other strains of this pathovar also showed the presence of one LOV-HK-RR protein. Regarding the presence of PHY-containing proteins, which might be involved in red light perception, the

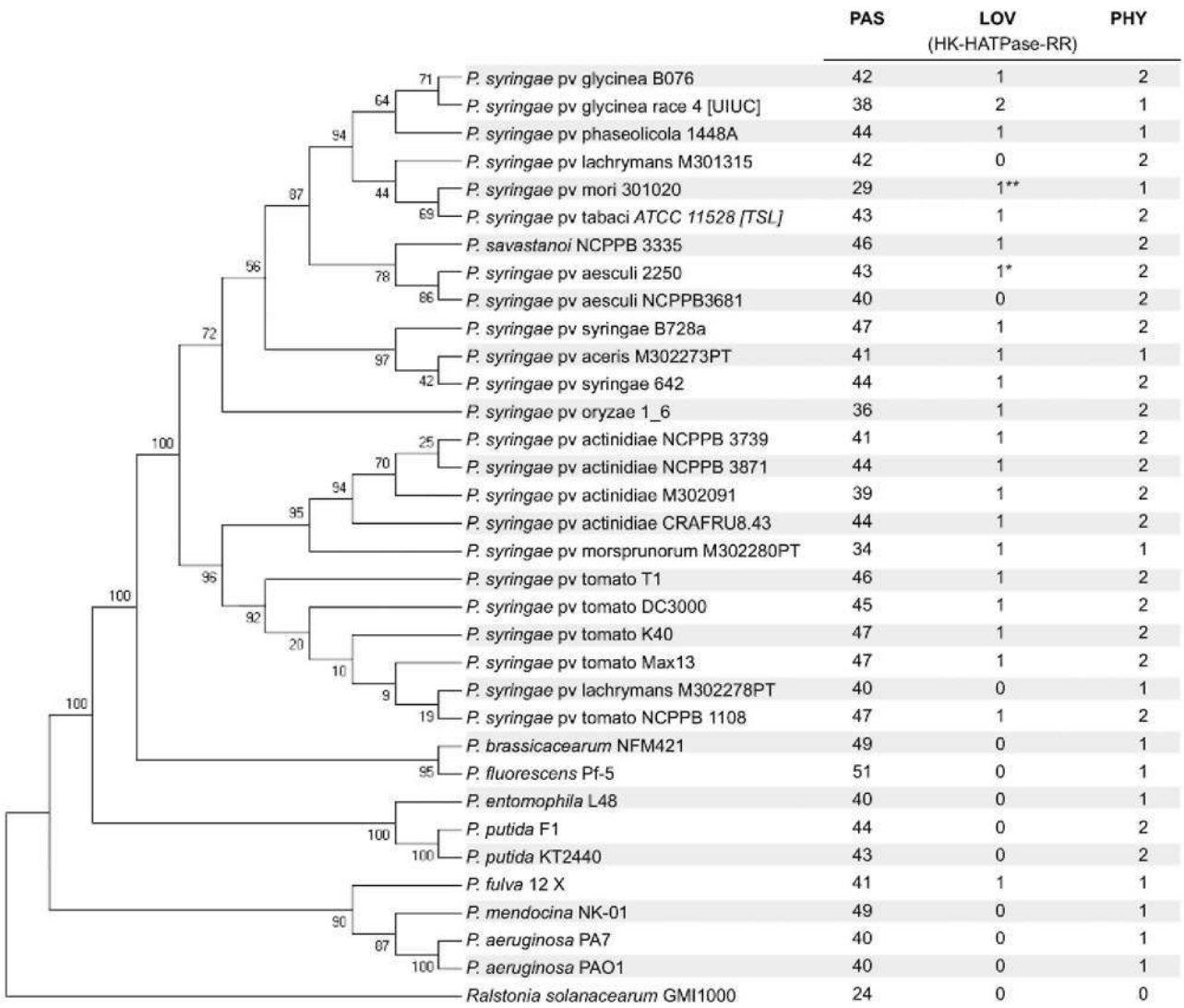

Fig. 1. Presence of photosensory proteins in *Pseudomonas* strains in a phylogenetic context. The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model. The percentage of trees in which the associated taxa clustered in the bootstrap test (500 replicates) is shown next to the branches. PAS, LOV, HK-HATPase, RR and PHY stand for per (period circadian protein) arnt (aryl hydrocarbon receptor nuclear translocator protein) sim (single minded protein); LOV; HK containing ATPase domain; response regulator; phytochrome; respectively. *The protein contains the module LOV-HK-HATPase but lacks the RR domain. **The protein contains the module LOV-HK but lacks the HATPase and RR domains. The *Ralstonia solanacearum* genome was included as an outgroup for the phylogenetic analysis.

number of putative bacteriophytochromes identified varied between one and two for most of the *P. syringae* phytopathogenic strains analysed and for soil plant-associated bacteria. Pto has two bacteriophytochromes.

White light and particularly the blue component inhibit bacterial motility and promote plant attachment

Pto exhibits a swarming phenotype on 0.3% agar plates, where flagellated bacteria typically develop surface-associated movement (Berti *et al.*, 2007). To determine the effect of white light on Pto swarming, the bacteria were cultured for 16 h under 15, 50, 55, 60 and 70 $\mu E\ m^{-2}\ s^{-1}$ white light or dark conditions (Fig. S1). Notably, light intensities generated through artificial indoor lighting are typically below 20 $\mu E\ m^{-2}\ s^{-1}$ in standard lab rooms, while the intensities are higher outdoors and in plant growth chambers (100–150 $\mu E\ m^{-2}\ s^{-1}$). A clear inhibition of motility was observed under white light intensities above 60 $\mu E\ m^{-2}\ s^{-1}$, whereas the characteristic-swarming behaviour described for Pto was observed at lower intensities and under dark conditions (Fig. S1). To reveal the particular contribution of the two major types of monochromatic light (i.e. blue and red light) to the inhibition of Pto motility, bacteria were

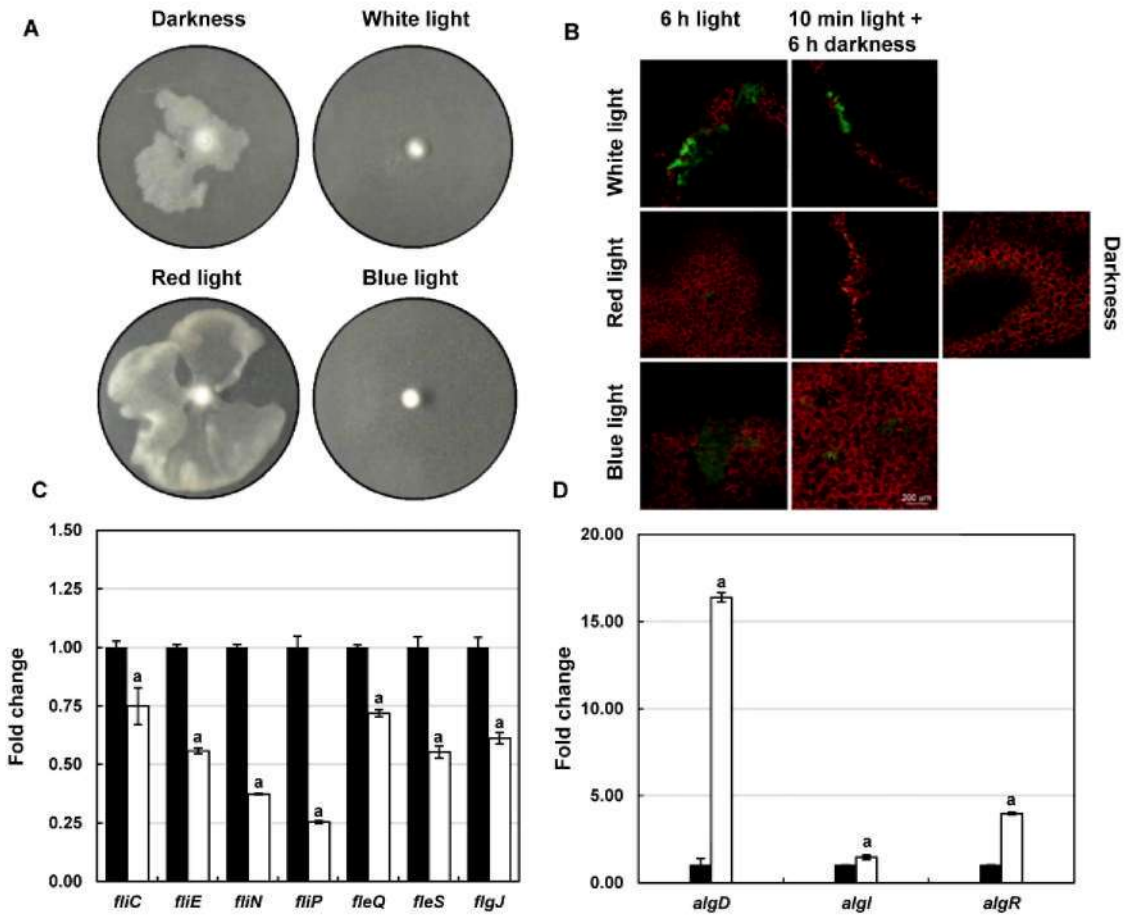

Fig. 2. Light conditions affect Pto swarming motility and attachment to plant leaves.
A. KB agar plates (0.3%) were inoculated with the WT strain using a sterile toothpick and incubated for 16 h at 28°C. The light conditions included: darkness, white light (70 μE m^{-2} s^{-1}), red light (20 μE m^{-2} s^{-1}) and blue light (20 μE m^{-2} s^{-1}).
B. 2×10^{7} cfu ml^{-1} was placed on the underside of *A. thaliana* leaves. The leaves were incubated for 6 h under constant light (white, red or blue), 10 min under light (white, red or blue), followed by 6 h of dark conditions or for 6 h in the darkness. Overlay of the GFP signal and chlorophyll autofluorescent confocal images is shown.
C. Differential expression of flagella and (D) alginate biosynthesis genes was evaluated by qRT-PCR after a 10 min white light treatment (white bars) with respect to the darkness (black bars). The means and standard errors of three replicates are shown. Error bars represent SEM (Standard Error Mean). (a) Significant differences between light and dark treatments were determined according to Student's *t* test ($P < 0.05$). The results shown in (A–D) are representative of at least three independent experiments.

grown under white (70 μE m^{-2} s^{-1}), red (20 μE m^{-2} s^{-1}) or blue (20 μE m^{-2} s^{-1}) light conditions. The data showed that the blue light component of white light is sufficient to inhibit Pto motility, and red light exerts the same effects as dark conditions facilitating bacterial motility (Fig. 2A).

Because light causes oxidative stress, which could have a toxic effect on bacteria, Pto growth was monitored for 24 h in both liquid and solid medium (Fig. S2) under the same lighting conditions described earlier. The results confirm that the photo-inhibition of Pto motility is not the consequence of an indirect effect on bacterial growth.

On the other hand, biofilm development is incompatible with the single cell motile stage, and light influences the switch between both stages in bacteria (Gomelsky and Hoff, 2011). As blue light exerts a potent inhibitory effect on motility, we hypothesized that blue light could signal the switch to a non-motile attached stage. To test this hypothesis, we assessed the attachment of a Pto-GFP (green fluorescent protein) strain to *Arabidopsis thaliana* leaves under different light conditions (Fig. 2B). We observed that after 6 h under white and specifically under blue light, the bacteria remained attached, forming organized structures, which could be considered as biofilms (although biofilm and attachment are not necessarily equivalent). Under dark conditions and red light, the remaining bacteria were not attached. Moreover, a 10-min light treatment before plant challenge was sufficient to induce the observed phenotypes regarding bacterial attachment (Fig. 2B).

White light controls expression of alginate biosynthesis genes and flagellar biogenesis

To get more insight about the effect of light on the observed phenotypes, we carried out qRT-PCR (quantitative real time-PCR) analyses of the expression of genes involved in motility and polysaccharide production. To analyse the expression of genes involved in flagellum biosynthesis, we selected *fliP* and *flgJ* that codify elements related with the biosynthesis, *fliC* that codifies flagellin, *fliN* and *fliE* that codify a flagellar motor switch protein, and the flagellar hook basal body, respectively, and finally *fleS* and *fleQ* involved in signal transduction and regulation of flagellar functions. The selection was based both on the differential functions reflected as well as on their genetic location in different transcriptional units. The data showed the downregulation by a 10-min white light treatment of these genes associated with the observed motility phenotype (Fig. 2C). Exopolysaccharides have been described to be involved in adhesion and resistance mechanisms during the epiphytic stage. Alginate is a Pto exopolysaccharide that contributes to virulence and epiphytic fitness (Yu *et al.*, 1999; Keith *et al.*, 2003). We chose *algI*, *algR* and *algD* for the expression analysis. AlgI and AlgD are involved in the alginate biosynthesis and were selected for being located in different operons. AlgR is a regulatory protein controlling alginate biosynthesis. We found that these genes were upregulated by a 10-min white light treatment (Fig. 2D).

Virulence is differentially regulated by blue and red light

As light controls motility and surface attachment in Pto, we examined whether the light also alters the virulence of this pathogen. Tomato plants, the host plants of Pto infection, were challenged with bacterial cells, pretreated or not with white light for 10 min before inoculation. Then, plants were transferred to a climate-controlled chamber under standard light cycle conditions (12 h light/12 h darkness), starting in the darkness. After 6 days, the symptoms and bacterial population were recorded. Figure 3A shows that both the symptoms (evaluated as number of chlorotic-necrotic spots) and bacterial population were reduced in plants challenged with bacterial cells pretreated with light.

Arabidopsis thaliana is also a host plant for Pto, so we performed the same experiment using this plant and observed the same differences after white light treatment as observed in tomato (Fig. 3B). To differentiate the effects of blue and red light on bacterial virulence, *Arabidopsis* plants were inoculated with bacterial cells subjected to the 10-min treatment under blue or red monochromatic lights. The results showed that blue light is responsible for the reduction of the symptoms, whereas red light generates the opposite effect, enhancing the appearance of symptoms compared with the darkness treatment.

To determine the biological significance of the light treatment in the control of the virulence, we designed a specific plant inoculation experiment. As the LOV photoreceptor dark recovery occurs within 5650 s (94.16 min) (Cao *et al.*, 2008), we performed a plant challenge experiment in which bacterial inoculum was subjected to a 10 min light/100 min darkness/10 min light cycle. After each treatment an aliquot of the bacterial suspension was used to inoculate *Arabidopsis* plants. Figure 3C shows the bacterial population at 6 days post-inoculation (dpi) in plants challenged with the different suspensions. The results show that after treatment in darkness for 100 min, virulence was restored compared with the inhibition attained after light treatment; a subsequent 10-min light treatment reduced the bacterial virulence again.

Light controls the entry to the plant apoplast

As both the motility phenotype and virulence are altered after white light treatment and it has been previously suggested the requirement of an active motility to enter the plant apoplast, we decided to further study the relevance of flagellar motility in the context of the entry process.

We first examined whether the amount of FliC, the flagellar filament protein, was altered. The presence of this protein in samples from bacterial cultures grown 6 h under white light and dark conditions was assessed through Western blot analyses (Fig. 4A and B). The apparent molecular weight of the lower band corresponds to the predicted size for the Pto flagellin (29 kDa). The upper band corresponds to the expected size of the glycosylated flagellin (32 kDa), as previously described (Takeuchi *et al.*, 2003). The results clearly showed that the amount of the non-glycosylated flagellin was reduced under light conditions. In addition, we stained the bacterial samples obtained from the border regions of the migration zones of swarming plates for flagella visualization. Figure 4C shows that under 16 h white light conditions, Pto cells practically do not present flagellar structures.

To test whether the repression of motility by white light is affecting the entry process and therefore the onset of the infection, we infiltrated *A. thaliana* leaves with Pto cells pretreated or not with white light for 10 min before inoculation. We compared two types of inoculation methods: spray inoculation versus infiltration under the premise that sprayed cells would need active motility to reach entry sites. The results showed that when infiltrated with Pto cells pretreated with white light, the differences regarding both symptoms and bacterial population in plant are smaller than those when using spray inoculation

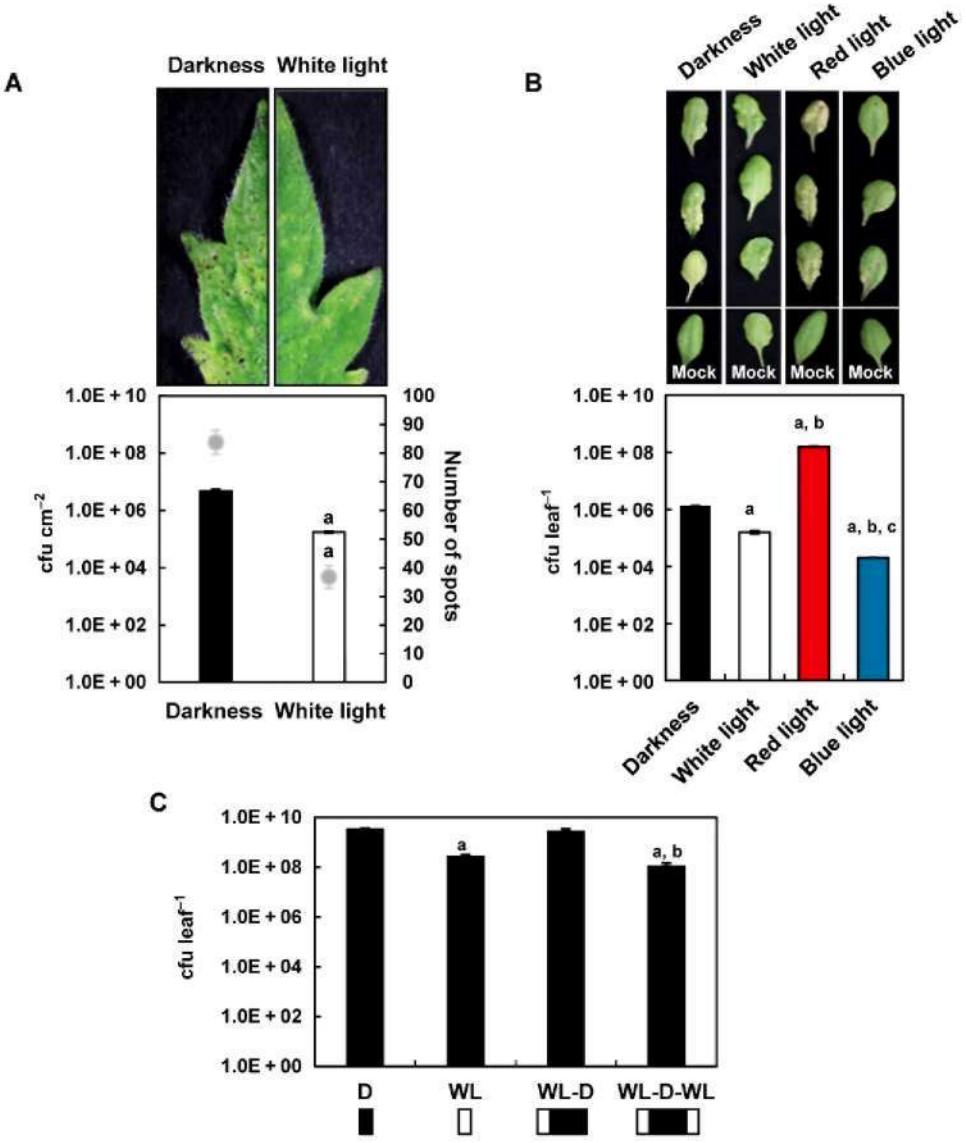

Fig. 3. Light treatment of Pto before infection reduces disease symptoms and bacterial population.

A. Bacterial population per unit area (bars) and number of lesions (grey points) on tomato plants were determined at 6 days post-inoculation (dpi) by dipping with suspensions of 10^8 cfu ml $^{-1}$ cells pretreated for 10 min with white light or maintained under darkness. The means and standard errors of five replicates are shown. (a) Significant differences between light and dark treatments were determined according to Student's *t* test ($P < 0.05$).

B. Disease symptoms and bacterial population (bars) were observed on *A. thaliana* Col-0 leaves at 6 dpi by spraying Pto cells suspensions containing 3×10^8 cfu ml $^{-1}$ pretreated for 10 min either with white light (70 µE m $^{-2}$ s $^{-1}$), red light (20 µE m $^{-2}$ s $^{-1}$), blue light (20 µE m $^{-2}$ s $^{-1}$) or maintained under darkness. The means and standard errors of three replicates are shown. Significant differences with respect to the dark (a), white light (b) and red light (c) treatments were determined according to Student's *t* test ($P < 0.05$).

C. Pto population in *A. thaliana* Col-0 leaves at 6 dpi by spraying Pto cells suspensions containing 3×10^8 cfu ml $^{-1}$. The bacterial suspension was pretreated for 10 min with white light (70 µE m $^{-2}$ s $^{-1}$) (WL) or maintained under darkness (D). The bacterial suspension treated with light was subsequently subjected to a 100-min dark treatment (WL-D) and treated again with 10 min of white light (WL-D-WL). After each treatment, an aliquot of the inoculum was used to challenge plants. The means and standard errors of three replicates (three leaves each) are shown. Significant differences with respect to the dark treatment (D) (a), and with respect to the second dark treatment (WL-D) (b) were determined according to Student's *t* test ($P < 0.05$). The results shown in (A–C) are representative of at least three independent experiments.

(Fig. 4D and results in Fig. 3B). A Pto *fliC* mutant strain was used to check the requirement of the flagellum during the entry process. The virulence of this strain when sprayed is significantly less virulent than the wild-type (WT) strain (Fig. 4E), while when using infiltration, the *fliC* mutant virulence is at the same level of the WT virulence (Fig. 4F). This reveals that an active movement is essential to enter the plant apoplast and to initiate the infection.

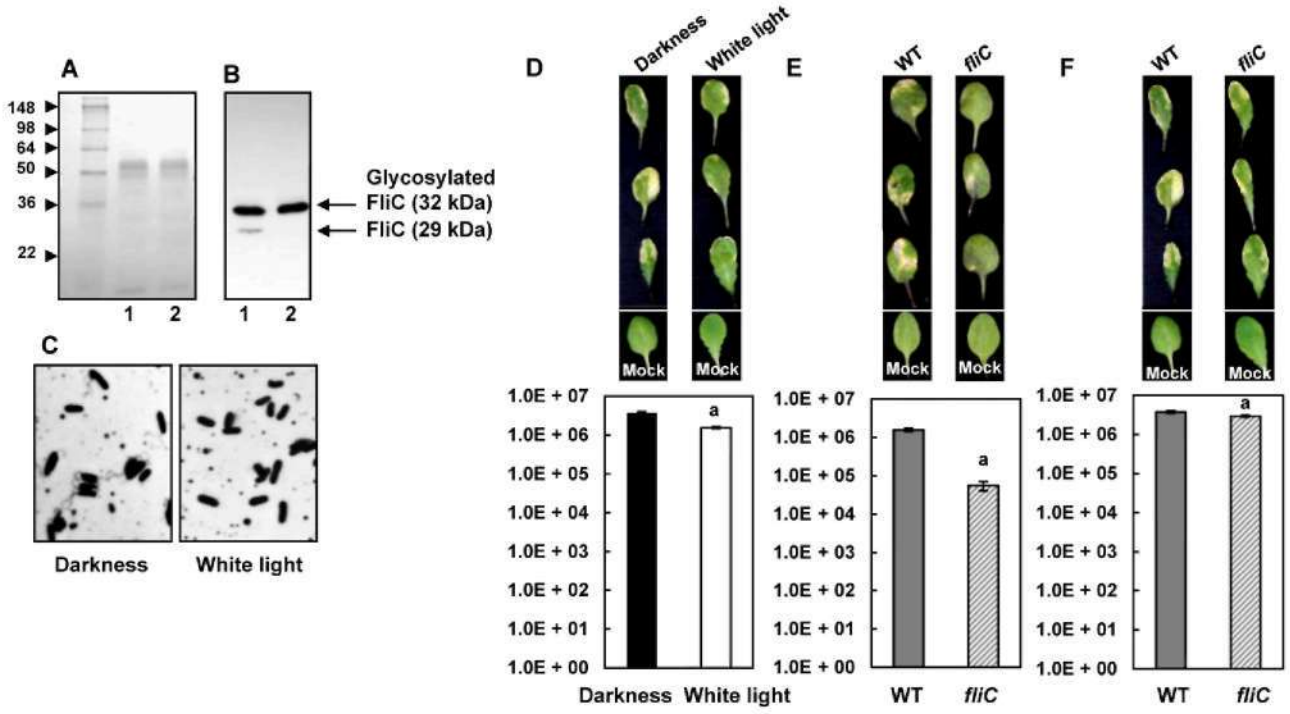

Fig. 4. White light controls the entry to plant apoplast mediated by an active motility.
A. Extracellular and membrane proteins from bacterial cultures exposed to darkness (lane 1) or white light (lane 2) were electrophoresed in 15% acrylamide gels and stained with Coomassie Brilliant Blue.
B. Western blot analysis of these proteins reacted with a flagellin antiserum. The observed bands running at approximately 29 and 32 kDa correspond to FliC and glycosylated FliC, respectively.
C. Images (630×) of bacteria stained with simplified Leifson method are shown.
D. Disease symptoms and bacterial population (bars) were observed on *A. thaliana* Col-0 leaves at 2 dpi by infiltrating Pto cells suspensions containing 10^5 cfu ml^{-1} pretreated for 10 min with white light (70 μE m^{-2} s^{-1}) or maintained under dark conditions. The means and standard errors of three replicates are shown. (a) Significant differences between light and dark treatments were determined according to Student's *t* test ($P < 0.05$).
E. Disease symptoms and bacterial population (bars) were observed on *A. thaliana* Col-0 leaves at 6 dpi by spraying Pto WT and *fliC* cells suspensions containing 3×10^8 cfu ml^{-1}.
F. Disease symptoms and bacterial population were observed on *A. thaliana* Col-0 leaves at 2 dpi by infiltrating Pto WT and *fliC* cells suspensions containing 10^5 cfu ml^{-1}. For (E) and (F), the means and standard errors of three replicates are shown. (a) Significant differences between Pto WT and Pto *fliC* treatments were determined according to Student's *t* test ($P < 0.05$). The results shown in (A)–(F) are representative of at least three independent experiments.

All together, these results suggest that the effect of light over Pto motility is the main responsible of the reduction in virulence observed in light-pretreated cells.

The ability to regulate light-related phenotypes is compromised in the LOV-Pto mutant

To determine the contribution of the LOV-Pto photoreceptor to the light-dependent phenotypes observed, a mutant in this gene was constructed. The swarming motility of this strain was assayed under different light conditions (Fig. 5A), and the results showed that neither white nor blue light completely inhibited the motility of the LOV mutant compared with the inhibition observed in the WT strain. However, the behaviour of this strain under red light conditions was not altered. The growth rate of LOV mutant in culture media was similar to that of the WT strain both under darkness and light conditions (data not shown). The phenotype was restored to WT levels in the complemented strain. These results suggest that this photoreceptor is partially responsible for the bacterial response under both white and blue light conditions. Moreover, the analysis of the expression of the *fliC* gene through qRT-PCR in the LOV-Pto mutant showed that there were no differences in the expression of this gene under light treatment or darkness (Fig. 5B).

The attachment of the LOV-Pto mutant strain to the plant surface was also evaluated in *Arabidopsis* leaves (Fig. 5C). While the WT strain attached leaves under light conditions, the mutant strain did not remain attached to the plant surfaces. The results suggest that this phenotype was also under the control of light perception through this photoreceptor. Moreover, through qRT-PCR analyses, we observed that under

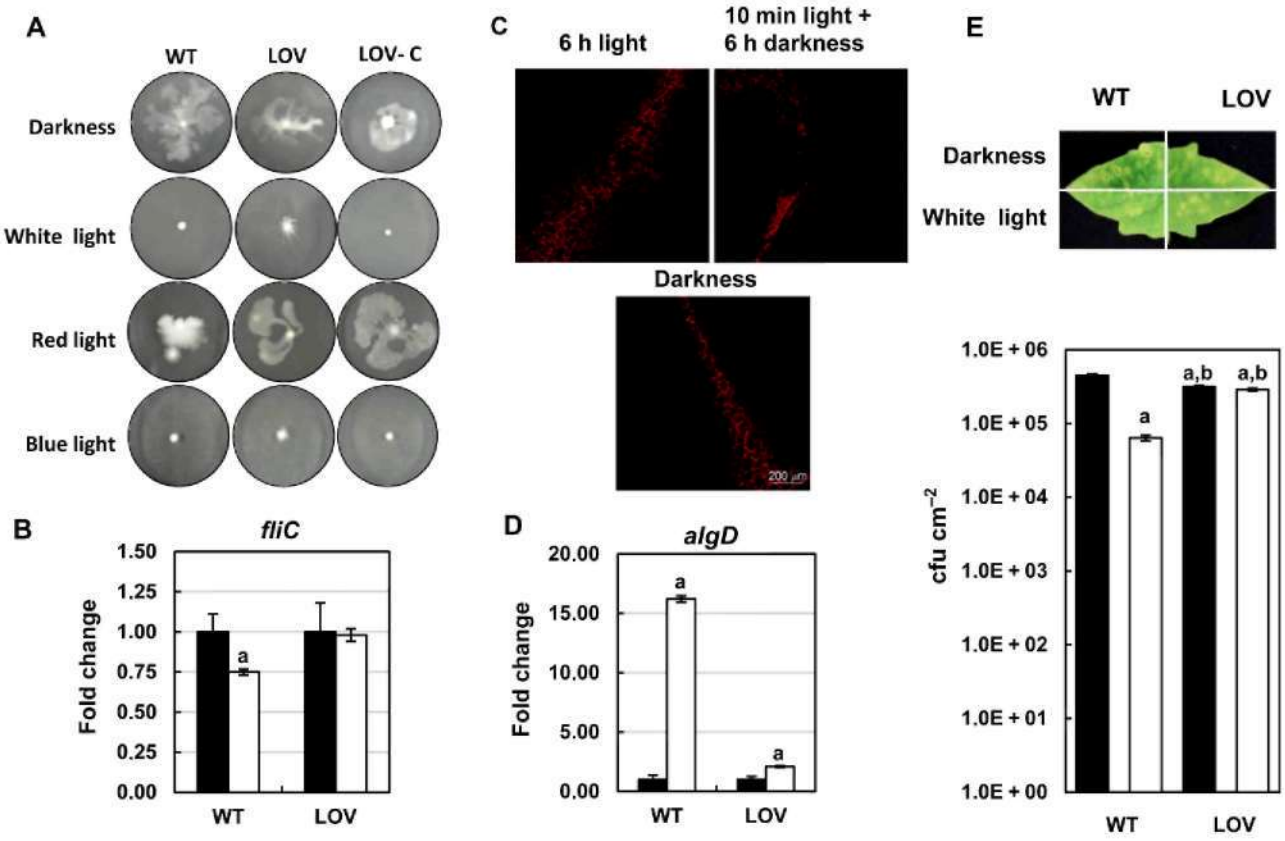

Fig. 5. LOV-Pto mutant response to light is altered.
A. Semi-solid KB agar (0.3%) plates were inoculated with Pto WT, LOV-Pto mutant and a LOV-C complement strain using a sterile toothpick. The plates were incubated for 16 h at 28°C under dark, white light (70 µE m^{-2} s^{-1}), red light (20 µE m^{-2} s^{-1}) or blue light (20 µE m^{-2} s^{-1}) conditions.
B. 2×10^7 cfu ml^{-1} of the LOV-Pto mutant was placed on the underside of *A. thaliana* leaves. The leaves were incubated for 6 h under constant white, red or blue light or for 10 min with white, red or blue light, followed by 6 h of dark conditions or for 6 h in the darkness. Overlay of the GFP signal and chlorophyll autofluorescent confocal images is shown.
C. Differential expression of *fliC* and (D) *algD* genes was evaluated by qRT-PCR after a 10-min white light treatment (white bars) or dark treatment (black bars) in a LOV-Pto mutant with respect to the Pto WT strain. The means and standard errors of three replicates are shown. (a) Significant differences between dark and light treatments were determined according to Student's *t* test ($P < 0.05$).
E. Disease symptoms and bacterial population (bars) were determined on tomato plants at 6 dpi by dipping with suspensions containing 10^8 cfu ml^{-1} of Pto WT and LOV-Pto mutant cells pretreated for 10 min with white light (70 µE m^{-2} s^{-1}) or maintained under darkness. The means and standard errors of five replicates are shown. Significant differences with respect to the dark treatment (a) and with respect to the white light treatment (b) of the WT strain were determined according to Student's *t* test ($P < 0.05$). The results shown in (A)–(E) are representative of at least three independent experiments.

light conditions, the expression of *algD* in the LOV-Pto mutant was much lower than the expression in the WT strain (Fig. 5D).

To analyse the virulence of LOV-Pto mutant, we challenged tomato plants with the WT and mutant strains after a 10-min treatment under dark or white light conditions. We observed that regardless of the light treatment, the mutant strain was less virulent than the WT strain for both symptom production and bacterial population at 6 dpi. Moreover, the virulence in the mutant strain was not regulated by white light, and the symptoms and bacterial population recorded *in planta* was not diminished after the white light treatment, as observed for the WT strain (Fig. 5E).

Discussion

Proteins with photo-reactive LOV or PHY domains involved in blue and red light perception, respectively, have been identified in phytopathogenic bacteria (Rottwinkel *et al.*, 2010; Bonomi *et al.*, 2012; Kraiselburd *et al.*, 2012). Our analysis of the distribution of the canonical LOV-HK-RR structure revealed the presence of at least one member of this family in almost all the plant-pathogenic *P. syringae* genomes analysed. Interestingly, the canonical blue light receptor is not present in root-associated *Pseudomonas* species (*P. putida* and *P. fluorescens*), suggesting that the evolutionary incentive to use blue light as a source of information primarily exists

among bacteria exposed to day light cycling conditions, as observed for most of the *P. syringae* strains analysed. These plant pathogens typically exhibit an epiphytic phase prior to the pathogenic phase (Hirano and Upper, 2000). In contrast with blue light receptors, phytochromes have been identified in many different strains, regardless of their niche. Indeed, these proteins (and specifically those classified as bathy phytochromes) have been described to dominate in *Rhizobiales* and are most frequently represented in soil bacteria (Rottwinkel *et al.*, 2010). The light regime in the soil is rich in long-wavelength light. Therefore, this is congruent with the idea that it might have an evolutionary incentive for the use of light as a source of information (van der Horst *et al.*, 2007), being red light more important for soil bacteria and white light more important for bacteria in the phyllosphere.

Therefore, the aim of the work is the study of the light effect on Pto during the stages previous to the infection, when this bacterium inhabits the phyllosphere (epiphytic stage) and how that influences the entry process, and subsequently the success of the infection. Of course, we do not discard a light effect during the interaction, but probably that effect is influenced by the plant response to the light.

During the course of this research, it became clear that the type, duration and intensity of the light treatments are critical to the phenotype regulation of Pto. Previous analyses of the light influence on different heterotrophic bacteria have been conducted using light treatments over long time periods (6–48 h), i.e. the experiments with Rle or Xax (Bonomi *et al.*, 2012; Kraiselburd *et al.*, 2012), or over shorter time periods (10 min to 1 h), i.e. experiments with non-plant pathogenic bacteria, such as *L. monocytogenes* (Ondrusch and Kreft, 2011) and *Brucella* spp. (Swartz *et al.*, 2007). The selection of the duration of the light treatment considered both the kinetics of the photocycle for the blue light photoreceptor present in Pto and the stress response associated with light conditions (Ziegelhoffer and Donohue, 2009). The time required for light activation of the LOV-photoreceptor and photoadduct formation, which initiates signal transduction, is short, at 20 ns and 1.5 μs, respectively. Whereas the recovery of the photoadduct to the dark state is slow, yielding an average lifetime of 5650 s (Cao *et al.*, 2008). Based on these considerations, we used a short light treatment of 10 min to further analyse the effects of light on different phenotypes or the gene expression that were not associated with long-term adaptive responses to light stress (oxidative stress, osmotic stress, etc.). The intensity of the white light used in our experiments (70 μE m^{-2} s^{-1}) is close to the illumination level at twilight on a day with clear sky conditions (Thorington, 1985). In contrast, the light intensity conferred by standard artificial indoor lighting is approximately 15 μE m^{-2} s^{-1}, while at midday on a sunny day at the coordinates 40°26′10″N 3°48′50″W, the intensity of the light outdoors is 1000 and 140 μE m^{-2} s^{-1} in a greenhouse.

Motility has been associated with bacterial infection of the host plant, as the entry process into plant apoplast depends on it (Panopoulos and Schroth, 1974; Ichinose *et al.*, 2003; Yao and Allen, 2006; Antunez-Lamas *et al.*, 2009). The results obtained in the present study showed that the blue, but not the red, component of white light is responsible for the inhibition of swarming motility in Pto. This feature is altered by light treatment in other plant-associated bacteria (Oberpichler *et al.*, 2008; Bonomi *et al.*, 2012; Kraiselburd *et al.*, 2012). For Atu, white light also inhibits motility, whereas for Rle and Xax, motility is not affected. Notably, the light intensities for Atu and in the present study are higher than those for Rle and Xax. Moreover, the gene expression analysis after the 10 min of treatment under these light conditions showed the downregulation of the genes involved in flagellar synthesis and function. These data were confirmed through Western blot experiments to detect the flagellar filament protein (FliC) and microscopic analyses of cells. The flagellar proteins are inhibited in the other models analysed (Oberpichler *et al.*, 2008; Bonomi *et al.*, 2012; Kraiselburd *et al.*, 2012). The analysis of motility and the *fliC* gene expression upon light treatment in the LOV-Pto mutant suggests that motility regulation depends on this photoreceptor. These results support the hypothesis that the blue component of the light inhibits flagella-dependent motility in Pto. Moreover, there are clear differences in the results obtained using an inoculation method that requires motility (spray) versus an inoculation method that does not (infiltration). The results using infiltration still reveals differences between the white light treatment and the dark treatment suggesting that motility is the main factor affected by the treatment, although other virulence determinants might be regulated by light at this stage. Actually, a Pto *fliC* mutant is severely affected in virulence when the inoculation method makes an active movement essential to enter the plant apoplast and initiate the infection. The enhanced virulence of a Pto *fliC* mutant observed when infiltrated could be due to the absence of flagellin recognition by plant. Actually, it has been described that plant immunity triggered by flagellin limits pathogen fitness (Cai *et al.*, 2011). Flagellar motility contributes to epiphytic fitness in *Pseudomonas* spp. (Haefele and Lindow, 1987), and very recently, Yu and colleagues (2013) have found that flagellar motility is an adaptive feature of the epiphytic phase in *P. syringae* pv syringae B728a. Our results suggest that flagellar motility, which is essential for spreading on the leaf and for the entry to the plant apoplast, is controlled by light. In this context, the active motility of bacteria on plant surfaces would be favoured under low-intensity light conditions.

On the other hand, biofilm formation and attachment to plant surfaces requires the inhibition of bacterial motility and, therefore, the switch from a motile state to a sessile state (Verstraeten *et al.*, 2008). The experiments using GFP-labelled bacteria showed that a light treatment as short as 10 min was sufficient to promote bacterial attachment to *Arabidopsis* leaves. Consistently, the qRT-PCR data showed that the genes involved in alginate synthesis were upregulated after light treatment. Moreover, the LOV-Pto mutant did not attach to *Arabidopsis* leaves and showed an altered expression of *algD* in response to light, suggesting that the blue component of white light is responsible for this phenotype. For *Rhizobiales*, including Atu and Rle, the adherence to biotic surfaces is inhibited by light, whereas for Xax, similar to Pto, blue light induces adherence to plant surfaces. Light might inhibit the transition from one state to another, but the particular regulation depends on the environmental significance of light for the bacteria in a given niche (Gomelsky and Hoff, 2011). These results are in line with our hypothesis that the switch from a motile to a sessile state is regulated by light in Pto.

The virulence of Pto (measured as symptom development and bacterial population) was diminished after the 10 min white and blue light treatments in both tomato and *Arabidopsis*. The reduced virulence could be the consequence of the downregulation of flagellar genes, which in turn causes a reduction in the motility-dependent entry process. Therefore, the results obtained in the present study suggest that light absence is an important factor for the transition from the pre-infection to the infection phase. A parallel phenomenon has been reported in Atu and Xax (Oberpichler *et al.*, 2008; Kraiselburd *et al.*, 2012). However, in the present study, we treated the cells prior to plant inoculation for 10 min with the desired light, while in previous Atu and Xax experiments, the plants were subjected to the light treatment after infection. Surprisingly, for Pto, red light treatment increases the bacterial virulence in *Arabidopsis* plants. It cannot be ruled out that the downstream regulation through white light perception reflects the balance between red and blue light. Further investigations regarding the signal transduction process under both types of light will be informative to unveil this aspect.

The LOV-Pto mutant is less virulent than the WT regardless of the light treatment. A similar result has been reported for the mutant in the Xax homologue protein (Kraiselburd *et al.*, 2012). However, we cannot rule out that this photoreceptor might be regulating other aspects of the bacterial physiology. In any case, these results show that the mutant loses the ability to regulate the response to the changing light conditions.

In this study, we conducted a comparative analysis of the available *Pseudomonas* genomes to identify proteins containing LOV and PHY domains. In addition, we studied the effect of light on Pto phenotypes like motility and adhesion, which in turn are related to virulence. We observed that the blue light component is primarily responsible for the observed phenotypes. This conclusion was confirmed through an analysis of the phenotype resulting from a mutation in the LOV protein involved in blue light perception.

In summary, this work demonstrated the key role of light perception in Pto phenotype switching. Several questions arise from these results: What is the relative importance of this environmental signal in the context of the plant–pathogen interactions? Does Pto use light as the master signal to regulate virulence? It is reasonable to propose that Pto uses this environmental signal not only as a direct warning of desiccation and oxidative stress but also as an indirect warning of the plant defence level. The knowledge of light-sensing mechanisms and the pathways involved in the control of phenotype switch will open new avenues for designing strategies to interfere with this phenomenon to prevent the establishment of bacterial infections.

Experimental procedures

Identification of protein domains

The genome sequences (including plasmids sequences when present) of 33 bacterial species belonging to the genus *Pseudomonas* were downloaded from the ASAP (A Systematic Annotation Package) website (http://asap.ahabs.wisc.edu/asap/home.php) and NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/). The identification of PHY and PAS protein domains was performed using an internal pipeline. First, PHY and PAS domains full alignments were downloaded from the PFAM website of the Sanger Centre (http://pfam.sanger.ac.uk/) using the PFAM identifiers PF00989 and PF00360, respectively. These alignments were used to build Hidden Markov Models (HMMs) using HMMER v2.3.2 (Eddy, 1998). Finally, a customized BioPerl (Stajich *et al.*, 2002) script combined with HMMER searched for HMM hits using an *E*-value of 0.005 (Tables S1 and S2). For the LOV domains, only PAS-containing proteins containing the amino acid sequence GXNCRFLQ (Briggs, 2007) within the PAS domain were selected. HisKA, HATPase and Response_reg domains were identified using the PFAM identifiers PF00512, PF02518 and PF00072, respectively. The *Ralstonia solanacearum* GMI1000 genome was included as an outgroup.

Phylogenetic tree

The phylogenetic analysis of the bacterial species was performed through multilocus sequence analysis using a concatenated data set of *gltA*, *recA*, *rpoA*, *gyrA* and *gyrB* genes. The phylogenetic tree was obtained using the Maximum Likelihood method based on the JTT (Jones-Taylor-Thornton) matrix-based model (Jones *et al.*, 1992). The percentage of trees in which the associated taxa clustered in the bootstrap test (500 replicates) is shown next to the branches in Fig. 1 (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically using Neighbor-Join and BioNJ

algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log likelihood value. The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated. A total of 2739 positions were included in the final data set. Multiple alignments and evolutionary analyses were conducted using MEGA5 software (Tamura *et al.*, 2011).

Microbiological methods and illumination conditions

The strains used in this study are described in Table S3. The Pto strains were grown at 28°C in King's B (KB) medium (King *et al.*, 1954). *Escherichia coli* strains were grown in Luria broth at 37°C. The antibiotics were used at the following final concentrations (μg ml^{-1}): ampicillin, 100; kanamycin, 20; gentamicin, 5; streptomycin, 20; rifampicin, 50. The bacterial growth was monitored at OD_{600} (optical density at 600 nm), using a Jenway 6300 spectrophotometer. White light from fluorescent tubes (cool daylight) was used at intensities of 15, 50, 55, 60 and 70 μE m^{-2} s^{-1}. Red and blue light from GreenPower LED HF red (660 nm) and GreenPower LED HF blue (470 nm) (PHILIPS, Amsterdam, the Netherlands), respectively, were used at an intensity of 30 μE m^{-2} s^{-1}. The effects of different colour lights on bacterial growth were assessed both in solid and liquid media. One millilitre of an exponential-phase (0.6 OD_{600}) culture was spread on the surface of 0.3% (wt vol^{-1}) KB agar plates. The plates were incubated at 28°C overnight under white (70 μE m^{-2} s^{-1}), red (20 μE m^{-2} s^{-1}) or blue light (20 μE m^{-2} s^{-1}), or maintained in darkness. Subsequently, the plates were carefully washed with 10 mM $MgCl_2$, and the bacterial growth was determined through serial dilution and plating. Pto was grown in liquid KB medium at 150 r.p.m. for 24 h at 28°C under the different light conditions as described earlier. Samples were collected every 90 min. For the Pto strains used in this study, an OD_{600} of 1.0 corresponded to 10^9 colony-forming units (cfu). Darkness conditions were examined using two different procedures with similar results: (i) plates and flasks were maintained in darkness covered with two layers of aluminum foil, and (ii) the plates and flasks were maintained in darkness inside a dark box. The temperature was monitored at all location for all experiments to ensure that the test conditions did not affect the temperature selected in the chamber.

DNA manipulation

The plasmids used in this study are described in Table S3. Briefly, to generate the LOV-Pto mutant, an internal 828 bp sequence of PSPTO_2896 (corresponding to nucleotides 40–868 of the coding sequence) from the Pto genome was amplified using Pfu DNA polymerase (Biotools, Madrid, Spain) and cloned into pKNG101 (Kaniga *et al.*, 1991). The resulting plasmid was transferred into Pto via triparental mating (de Lorenzo and Timmis, 1994). As pKNG101 cannot replicate in Pto, single crossover integrants were selected through streptomycin resistance. Plasmid integration was confirmed through PCR and Southern blot analyses.

For mutant complementation, the PSPTO_2896 gene was amplified from the Pto genome using primers that included the four basepair sequence (CACC) at the 5′ end of the forward primer necessary for the directional cloning into pENTR™/SD/D TOPO (Invitrogen, Carlsbad, CA, USA). The expression clones were generated through the recombination of the pENTR plasmid with the pCPP5040 expression vector using LR clonase (Invitrogen). The recombination reactions were performed according to the manufacturer's instructions. All expression constructs were confirmed through sequence analysis. The resulting plasmid was transferred into Pto via triparental mating (de Lorenzo and Timmis, 1994).

Swarming motility assays

The Pto strains were grown in KB medium to the exponential phase (OD_{600} = 0.5) under dark conditions. Subsequently, the cultures were centrifuged for 10 min at 6000 *g* at room temperature, and the cell pellets were homogenized with a sterile toothpick. Soft KB agar plates (0.3% wt vol^{-1}) were inoculated using a toothpick and incubated for 16 h at 28°C under different white light illumination conditions (15, 50, 55, 60 and 70 μE m^{-2} s^{-1}) or covered with two layers of aluminum foil under dark conditions. For the light-colour phototaxis assays, the plates were inoculated and incubated, as described earlier, under white (70 μE m^{-2} s^{-1}), red (20 μE m^{-2} s^{-1}) or blue light (20 μE m^{-2} s^{-1}) or covered with two layers of aluminum foil for dark conditions. Six plates for each treatment were incubated in three independent experiments.

Leaf attachment and confocal microscopy

For the leaf attachment assays, the Pto strains were grown for 24 h in KB agar plates under dark conditions. The cells were scraped from the plates and suspended in 10 mM $MgCl_2$ at a final concentration of 10^8 cfu ml^{-1}. A 200 μl drop was placed on the underside of the detached leaves from 3 week old *A. thaliana* plants. This process was conducted in dark conditions. Subsequently, three different experiments were set up: three replicates were incubated for 6 h under constant light (white, blue or red), three replicates were incubated for 10 min under light (white, blue or red), followed by 6 h under dark conditions, and three replicates were incubated for 6 h in the darkness. Before confocal microscopy, the leaves were washed twice through immersion in 10 mM $MgCl_2$. The GFP signal and chlorophyll autofluorescence were collected on a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) using laser lines of 488 and 633 nm, respectively. The same gain and offset settings were used for the different treatments. The images were processed using the LAS AF Lite 3.1.0 (Leica Microsystems). Three leaves were inoculated for each treatment in three independent experiments.

Plant bioassays

For plant inoculations, the Pto strains were grown for 24 h on KB agar plates under dark conditions. The cells were scraped from the plates and suspended in 10 mM $MgCl_2$ at the desired final concentration for each assay. This process was conducted under dark conditions (less than 0.05 μE m^{-2} s^{-1}). Prior to inoculation, the bacterial suspensions were either subjected to light treatment (white, red or blue light) for

10 min or maintained in the darkness. For tomato infection assays, Silwet L-77 at a final concentration of 0.02% vol/vol was added to the bacterial cell suspensions. All plants were incubated in a growth chamber at 25°C with 12 h light (100 $\mu E\ m^{-2}\ s^{-1}$) and a relative humidity of 60%.

For the virulence assays in tomato, 10 3 week old tomato plants (*Solanum lycopersicum* cv Moneymaker) were inoculated by dipping for 30 s into a bacterial suspension containing 10^8 cfu ml^{-1}. The symptoms were recorded at 6 dpi. The numbers of lesions on five equivalent tomato leaflets (with regard to age and location on plants) were counted. Bacterial growth in tomato leaves was measured by determining the average number of cfu isolated from five infected leaves. The plant material was homogenized in 10 mM $MgCl_2$, and serial dilutions were plated on KB solid medium containing rifampicin.

For virulence assays in *Arabidopsis*, 4 week old plants of *A. thaliana* Col-0 were inoculated. For the spraying assays, 25 plants were inoculated for each treatment with a suspension containing 3×10^8 cfu ml^{-1} and three plants were mock-sprayed with 10 mM $MgCl_2$. The symptoms were recorded at 6 dpi. For the infiltration assays, 25 leaves were infiltrated with a suspension containing 10^5 cfu ml^{-1}, and three leaves were mock-infiltrated with 10 mM $MgCl_2$. The symptoms were recorded at 2 dpi. In both kinds of experiments, bacterial growth on the leaves was determined by measuring the average number of cfu isolated from three leaves. The plant material was processed as described earlier. To assess the dark recovery of virulence, the same bacterial suspension was subjected to a 10 min white light treatment, followed by a 100 min darkness treatment and subsequently treated again with 10 min of white light. After each treatment, an aliquot of the inoculum was used to challenge the plants as described earlier. All plant bioassays were conducted three times.

RNA isolation and qRT-PCR analysis

Flasks with 40 ml of KB were inoculated with Pto cells to $OD_{600} = 0.05$ and grown with shaking at 200 r.p.m. at 28°C under dark conditions to $OD_{600} = 0.5$. The sample was split into two. Twenty millilitres were incubated for 10 min under white light with an illuminance of 70 $\mu E\ m^{-2}\ s^{-1}$, and the other 20 ml were covered with two layers of aluminum foil for dark treatment. RNA from three biological replicates from each treatment were obtained and cleaned with RNAeasy kit (QIAGEN, Venlo, the Netherlands). RNA was quantified using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA) and quality checked using an Agilent Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). Bacterial RNA (1 μg) was used for cDNA synthesis using random hexamers with the high-capacity cDNA reverse transcription kit, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Specific primers were designed to amplify fragments of approximately 100 bp for all genes (Table S4) using Primer3Plus. The *rpoD* gene, which has been previously described as a housekeeping gene for *P. syringae* (Sawada *et al.*, 1999), was chosen as an internal standard. The qRT-PCR was performed in an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems) using a SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. The following thermocycling conditions were used: 1 cycle at 95°C for 10 min; 50 cycles at 95°C for 15 s and 60°C for 1 min; and 1 cycle at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. The relative expression ratio was calculated as the differences between the cycle threshold (Benjamini *et al.*, 2001) values using the equation $2^{-\Delta\Delta Ct}$ as previously described (Pfaffl, 2001; Rotenberg *et al.*, 2006). A melting curve was performed at the end of each assay to certify the absence of primer-dimers and the presence of a single PCR product.

Protein extraction and Western blots

Flagellin from Pto cultures incubated 6 h under white light or dark conditions was purified, as previously described (Capdevila *et al.*, 2004). The preparations were separated on 15% SDS/PAGE (sodium dodecyl sulfate/Polyacrylamide gel electrophoresis) and stained with Coomassie Brilliant Blue R-250. The same electrophoretic conditions were used for the Western blot analysis. Flagellin antiserum (anti-FliC) was used at 1: 20 000. The serum is an anti-FliC polyclonal antibody raised against the *P. fluorescens* F113 FliC protein.

Bacterial flagella stain

Flagella of bacteria taken directly from swarming plates incubated at 28°C overnight under white light (70 $\mu E\ m^{-2}\ s^{-1}$) or dark conditions were stained as previously described (Clark, 1976). Samples were examined with the oil immersion objective in an optical microscope (Axiophot, Zeiss, Jena, Germany).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Pto swarming motility depends on light intensity. KB agar plates (0.3%) were inoculated with the WT strain using a sterile toothpick. The plates were incubated for 16 h at 28°C under different light conditions. Similar results were obtained in three independent experiments.

Fig. S2. Different light conditions do not affect Pto bacterial growth. The light conditions included are: darkness, white light (70 $\mu E\ m^{-2}\ s^{-1}$), red light (20 $\mu E\ m^{-2}\ s^{-1}$) and blue light (20 $\mu E\ m^{-2}\ s^{-1}$). (A) Bacterial growth curves in KB liquid medium. (B) Bacterial population on KB soft-agar plates recorded after an overnight incubation. The values are the means and standard errors of three replicates. Similar results were obtained in three independent experiments.

Table S1. Bioinformatics search for photosensory proteins among *Pseudomonas*. Bioinformatics search for LOV, HK, HATPase and RR domains among *Pseudomonas* genomes using Hidden Markov Models. Gene ID from the ASAP database except for (*) which corresponds to the NCBI database.

Table S2. Bioinformatics search for bacteriophytochromes among *Pseudomonas*. Bioinformatics search for PHY domains among *Pseudomonas* genomes using Hidden Markov Models. Gene ID from the ASAP database except for (*) which correspond to the NCBI database.

Table S3. Bacterial strains and plasmids used in this study.

Table S4. qRT-PCR primers used in this study.